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SHORT COMMUNICATION

Pheromone dynamics in virgin and mated females of the sexually cannibalistic orb-web spider *Argiope bruennichi* (Araneidae)

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Abstract. Female chemical signals are crucial for sexual communication in spiders and convey detailed information about females' mating status or age. However, evidence for chemical communication in spiders is largely behavioral. Only a few spider pheromones are known and quantitative chemical analyses of individual spiders are especially scarce. In the European wasp spider, *Argiope bruennichi* (Scopoli, 1772), females emit the volatile trimethyl methylcitrate, which attracts males from a distance. A recent study on the role of this pheromone in close-range male mate choice revealed that older virgin females approaching oviposition produce higher amounts of pheromone than younger ones, thereby increasing their attractiveness to males, and stop pheromone signaling shortly after copulation. Here we provide more detailed analyses of temporal dynamics in pheromone production in both virgin and mated female *A. bruennichi*. Our results corroborate previous observations indicating that male mating behavior in this species is guided by variation in female signaling effort.

Keywords: Temporal variation, strategic signaling, pheromone quantification, gas chromatography-mass spectrometry
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Pheromones are specific chemical substances or substance mixtures that evolved for intraspecific communication (Karlson & Butenandt 1959) and are arguably the most ancient and widespread form of information transfer in animals (Wyatt 2014). Especially in arthropods, pheromones represent the predominant means of communication (Greenfield 2002). In the context of sexual communication, pheromones have the potential to convey a wealth of information for mate assessment and choice, including mating status, age, condition, and relatedness (Johansson & Jones 2007; Wyatt 2014).

As in other arthropods, sexual communication in spiders is predominantly chemical and numerous studies demonstrate that female chemical cues can convey fine-tuned information (Gaskett 2007; Trabalon & Bagnères 2010; Schneider & Andrade 2011). Males of many taxa discriminate between mated and virgin females (e.g., Gaskett et al. 2004; Roberts & Uetz 2005; Stoltz et al. 2007; Baruffaldi & Costa 2010, 2014; Sentenska & Pekár 2019). However, in some species, including, for instance, the funnel-web spider, *Agelenopsis aperta* (Gertsch, 1934) (Agelenidae) (Riechert & Singer 1995), the brown widow spider, *Latrodectus geometricus* C. L. Koch, 1841 (Theridiidae) (Waner et al. 2018), and the two wolf spiders *Schizocosa malitiosa* (Tullgren, 1905) (Baruffaldi & Costa 2010) and *Schizocosa ocreata* (Hentz, 1844) (Lycosidae) (Roberts & Uetz 2005), males can further discriminate between young and old females without direct contact, e.g., by female silk only. In all species, males generally prefer older virgin females over younger ones (see Fischer 2019 for a comprehensive review). This preference is adaptive, as in spiders a female's body mass is an important determinant of fecundity (Marshall & Gittleman 1994) and generally increases with age due to adult feeding and egg maturation. Moreover, older females are more likely to survive until egg laying, given a constant mortality rate (Rittschof 2011). In *S. malitiosa*, Baruffaldi et al. (2010) suggested that the increased attractiveness of silk from older females is due to the higher abundance of a putative contact pheromone on their silk compared to silk from freshly molted females. However, in most cases the nature and exact function of putative chemical signals remain elusive. Only a few spider pheromones have been chemically identified (Schulz 2013; Fischer 2019) and even for those that are known, we largely lack quantitative chemical analyses.

In the European wasp spider, *Argiope bruennichi* (Scopoli, 1772), sexual attractiveness of virgin females increases with age, but is greatly reduced after a single copulation (Schulte et al. 2010; Cory & Schneider 2016; Weiss & Schneider 2022). *Argiope bruennichi* is one of those few spider species for which a female sex pheromone has actually been identified. Virgin females produce the volatile trimethyl (2*R*,3*S*)-methylcitrate (and minor proportions of the (2*S*,3*S*)-stereoisomer), which attracts males from a distance (Chinta et al. 2010). Recently, we could demonstrate that older virgin females strategically increase their investment in pheromone signaling by producing significantly more pheromone than relatively younger ones, while all females stop pheromone signaling shortly after their first copulation (Weiss & Schneider 2022). Female *A. bruennichi* produce only two egg sacs on average (Schneider et al. 2005) and virgin females often produce a first batch of eggs about three weeks after their adult molt. Hence, the increased investment in male attraction by virgin females approaching oviposition is adaptive. However, the exact pattern of pheromone signaling over the course of a female's adult life is unknown. We close this gap by providing a detailed analysis of the temporal dynamics of pheromone signaling in female *A. bruennichi*, including the effect of mating on pheromone signaling.

We collected subadult *A. bruennichi* from different natural meadows in and around Hamburg, Northern Germany, over the course of three years from 2019 to 2021. In each year, we collected spiders before the start of the mating season in late June or early July and housed them in our laboratory at the University of Hamburg. We kept spiders individually under upturned plastic cups (250 or 500 ml depending on a spider's size) with a hole in the bottom stuffed with cotton wool under natural light conditions, a constant temperature of 25° C, and a relative humidity of 50 %. We provided spiders with water from a sprayer at least six days a week and, depending on their size, fed them with *Drosophila* sp. or *Calliphora* sp. twice a week. We checked spiders for molts daily and noted the date of the adult molt. We define a spider's age as days since adult molt.

To assess variation in pheromone production over time, adult virgin females were kept alive for 1 to 14 days after maturation (total $n = 175$; average n per day = 10). At a given day, we weighed females to the nearest 0.1 mg on a calibrated scale (Mettler Toledo AB54-S;

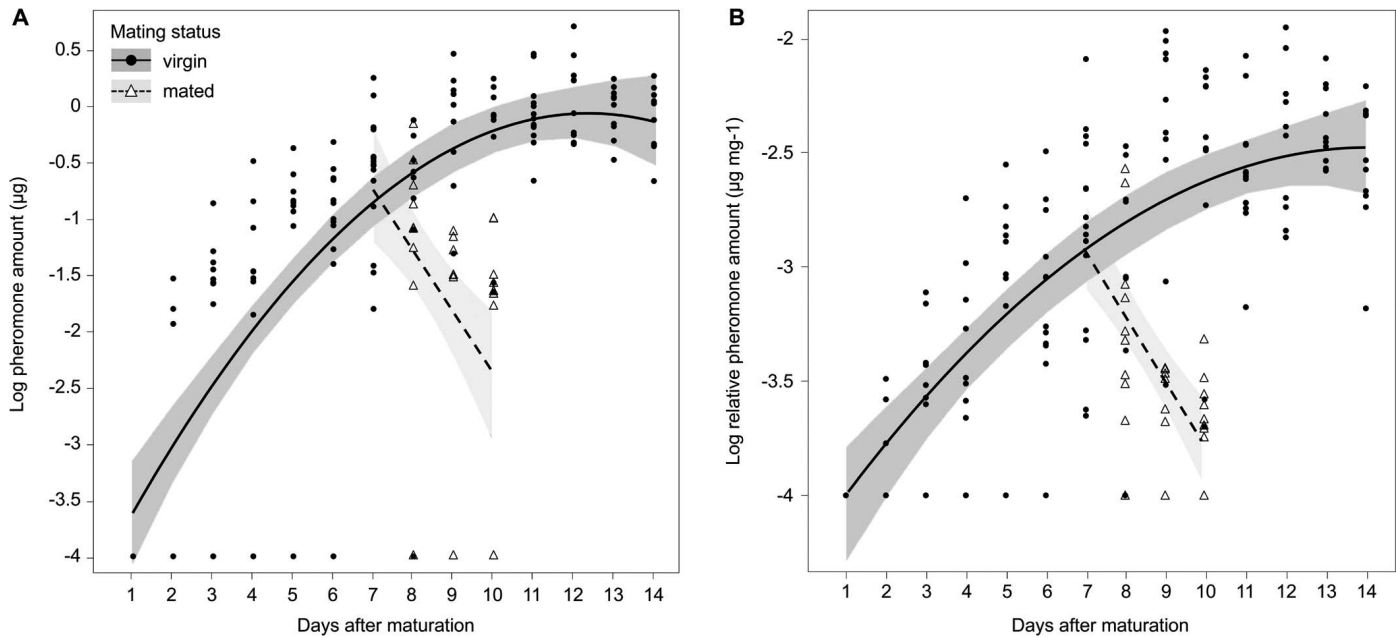


Figure 1.—Least squares regressions of log-transformed absolute pheromone amount (A) and log-transformed relative pheromone amount per unit body mass (B) in virgin (filled circles, solid line) and mated (open triangles, dashed line) females against female age (Note that negative log-values result from absolute pheromone amounts $< 1 \mu\text{g}$). Shaded areas give confidence regions.

Mettler Toledo LLC, Columbus, OH, US) and sacrificed them by freezing at -80°C . To assess the effect of mating on female pheromone signaling, we mated 30 females once with a randomly chosen male at the seventh day after maturation. The evening before a mating, we placed females into Perspex frames ($35 \times 35 \times 6 \text{ cm}$) where they could build their typical orb-webs overnight, which are necessary for male courtship and mating. We observed all matings and prevented females from cannibalizing the male, thereby excluding possible effects of cannibalism on pheromone signaling. To prevent a second mating, we removed surviving males immediately after copulation. We kept mated females alive for 1, 2, or 3 days after mating (10 females each) and then sacrificed them as described above.

To quantify the amount of the female pheromone trimethyl methylcitrate, we analyzed cuticular extracts of females by gas chromatography-mass spectrometry (GC-MS). We performed the chemical analyses in the same year we obtained the samples following the same protocol. We extracted individual females in 2 ml dichloromethane (GC-MS grade, Merck KGaA, Darmstadt, Germany) containing known quantities of octadecane (2019: $5 \mu\text{g}$, 2021 and 2022: $2.5 \mu\text{g}$; VWR, Darmstadt, Germany) as an internal standard. The suitability of octadecane as the internal standard for quantifying trimethyl methylcitrate has been established previously (Weiss & Schneider 2022). We concentrated extracts to approximately $90 \mu\text{l}$ and analyzed an aliquot of $1 \mu\text{l}$ by a Shimadzu GCMS-QP2010S system (Shimadzu Corporation, Kyoto, Japan), equipped with a SH-Rtx-5MS fused silica capillary column ($30 \times 0.25 \text{ mm ID}$, $0.25 \mu\text{m}$ film thickness; Shimadzu Corporation, Kyoto, Japan). We programmed the GC from 80 to 260°C at a constant rate of $30^{\circ}/\text{min}$ and from 260 to 300°C at a constant rate of $5^{\circ}/\text{min}$, with a 1-minute initial isothermal and a 5-minute final isothermal hold. A split-splitless injector was operated at a temperature of 250°C in the splitless mode. Helium was used as carrier gas at a constant flow rate of $1 \text{ ml}/\text{min}$. The ionization voltage of the electron ionization mass spectrometer was 70 eV . Source temperature was 200°C and interface temperature was 280°C . Data acquisition and storage were performed with the software GCMSsolution (Version 4.45; Shimadzu Corporation, Kyoto, Japan). We obtained peak areas by manual integration using

the GCMSsolution software. We identified the pheromone peak by comparing its characteristic mass spectrum and retention time to data from previous investigations (e.g., Chinta et al. 2010; Weiss & Schneider 2022).

We calculated absolute pheromone amounts (sum of the two stereoisomers) using the internal standard peak, as well as relative pheromone amounts per unit body mass by dividing absolute pheromone amount by body mass at the test day. Pheromone amounts showed highly unequal variances and were log-transformed before statistical analysis. Since many females produced no pheromone and log-transformation cannot be applied to zero values, we added the value of 0.0001 to all measured values before transformation. To compare pheromone amounts over time, we performed least squares regressions in PAST Version 3.25 (Hammer et al. 2001).

In virgin females, both absolute and body mass-normalized relative pheromone amounts showed a strong positive correlation with age that was best represented by second order polynomial regressions (Absolute amounts: $R^2 = 0.569$, $P < 0.0001$, $n = 145$; relative amounts: $R^2 = 0.562$, $P < 0.0001$, $n = 145$; Fig. 1). Females younger than three days rarely produced pheromone, while the amount of pheromone steadily increased from day 4 to day 8 and showed a marked increase in females older than 9 days, after which it remained more or less constant (Fig. 1, Supplementary Fig. S1, online at <https://doi.org/10.1636/JoA-S-22-005.s1>). In females that had been mated on the seventh day after maturation, absolute and relative pheromone amounts showed a negative linear correlation with age (Absolute amounts: $R = -0.523$, $P = 0.0002$, $n = 45$; relative amounts: $R = -0.680$, $P < 0.0001$, $n = 45$; Fig. 1). Mated females showed decreased absolute and relative pheromone amounts already 24 h after copulation and did not produce pheromone 48 h after mating (Fig. 1, Supplementary Fig. S1).

This study provides the first detailed analysis of changes in pheromone signaling over time in female spiders. Both absolute amounts of pheromone and relative amounts normalized by body mass increased considerably over the course of a female's adult life. Particularly the significant increase in mass-normalized pheromone

amount is crucial as it demonstrates a constantly increasing signaling effort, rather than just a passive accumulation over time or with increasing abdominal volume/surface in females close to oviposition (Weiss & Schneider 2022). These results substantiate our recent findings that mated female *A. bruennichi* stop pheromone production within two days after a single copulation and older virgins increase their pheromone production as compared to younger ones, which underlies their increased attractiveness for males (Weiss & Schneider 2022; see also Cory & Schneider 2016). Furthermore, females did not produce pheromone on the first two days after maturation and we detected only minute amounts in few three-day-old individuals, which explains observations from Schneider et al. (2016) showing that males are not able to discriminate between subadult and virgin adult females if the latter are younger than three days.

While we knew that older virgin *A. bruennichi* produce more pheromone, the almost five-fold increase in both absolute and relative amounts between day eight and nine is surprising. A field study observing mating rates and adult sex ratios in a natural population of *A. bruennichi* in northern Germany showed that the number of available adult males increases sharply at the beginning of a mating season, but noticeably decreases again after only ten days (Zimmer et al. 2012). As most females in these populations mature within the first few days of a mating season (Zimmer et al. 2012), this may represent an adaptation to the rapidly declining number of available males to increase mating chances in those females who have not secured at least one mating during the peak of the short mating season. Yet, in the above study, the number of roving males was always higher than the number of virgin females (Zimmer et al. 2012). Moreover, preliminary field observations in northern European populations suggest that many, if not most females mate within the first two days after maturation (E. Kramps, K. Weiss, J. M. Schneider 2021, personal observation; see also Schulte et al. 2010). A considerable proportion of females even receives at least one copulation while they molt to adulthood (Uhl et al. 2015). It remains an open question how males find subadults and young virgins. Taken together, the apparent scarcity of older unmated females (i.e., the rather low risk of remaining virgin more than eight days after maturation) leaves the question how such a marked increase in signaling effort in old females has evolved. A comparison with *A. bruennichi* populations in southern Europe might be revealing here. *Argiope bruennichi* has undergone a massive range expansion during the last decades and only recently colonized northern Europe, including northern Germany, probably due to adaptive introgression, and facilitated by global warming (Kumschick et al. 2011; Sheffer et al. 2021). The mating season at the northern range of the species' distribution is very short with a peak mating period of about two weeks (Zimmer et al. 2012). In the warmer Mediterranean climate zones of the species' original distribution range, however, adult molts may be less synchronized. This would prolong the mating season and potentially affect the effective sex ratio and thus a female's risk of remaining unmated.

Finally, the scatter of measured pheromone amounts in this study is high, especially in older females (see Fig. S1). However, this does not mean that these differences are not biologically relevant, as even small differences in pheromone amount between individual females might decide mating or reproductive failure. While the general ability of male *A. bruennichi* to sense differences in female pheromone amount has been demonstrated (Cory & Schneider 2016; Weiss & Schneider 2022), a next important step will therefore be to define the detection sensitivity and limit of males. Only this knowledge will allow a full evaluation of the effectiveness of increased pheromone signaling effort and possible benefits for females.

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SUPPLEMENTAL MATERIALS

Figure S1.— Absolute and relative pheromone amounts per unit body mass extracted from individual virgin and mated *Argiope bruennichi* females. Online at <https://doi.org/10.1636/JoA-S-22-005.s1>

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